Published in final edited form as:

Anal Chem. 2010 November 15; 82(22): 9543–9548. doi:10.1021/ac102330k.

Quantitative Measurement of Urinary Excretion of 3-Hydroxyisovaleryl Carnitine by LC–MS/MS as an Indicator of Biotin Status in Humans

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Abstract

Abnormally increased urinary excretion of 3-hydroxyisovaleryl carnitine (3HIA-carnitine) results from impairment in leucine catabolism caused by reduced activity of the biotin-dependent enzyme 3-methylcrotonyl-CoA carboxylase. Accordingly, urinary 3HIA-carnitine might reflect biotin status. Here, we describe an LC–MS/MS method for accurately quantitating the urinary concentration of 3HIA-carnitine at concentrations that are typical for excretion rates that are normal or only modestly increased. This method allows for high sample throughput and does not require solid-phase extraction. We used this method to provide evidence validating urinary 3HIA-carnitine as a biomarker of biotin deficiency in humans. Four healthy adult subjects were successfully made marginally biotin deficient by feeding a 30% egg white diet for 28 days. From study day 0 to 28, the mean urinary excretion of 3HIA-carnitine increased 3.5-fold ($p = 0.026$). These preliminary results indicate that urinary excretion of 3HIA-carnitine increases with marginal biotin deficiency. If these results are confirmed in studies involving larger numbers of subjects, urinary excretion of 3HIA-carnitine may potentially be a clinically useful indicator of biotin status.

Marginal degrees of biotin deficiency are teratogenic in several species.^{1–5} Marginal biotin deficiency in mice causes birth defects including cleft lip, cleft palate, and limb shortening defects with teratogenic rates approaching 100%.⁶ In both the timing and the degree of severity, biotin deficiency in human gestation resembles the mouse models of pregnancy. Accordingly, concern has been raised about the potential for human teratogenesis⁷ and the need for robust, valid indicators of marginal biotin deficiency in humans.⁸

Evidence indicating that marginal biotin deficiency does occur during the first trimester of human gestation include reduced activity of the biotin-dependent enzyme propionyl-CoA carboxylase (PCC) in peripheral blood lymphocytes (PBLs)⁹ and increased urinary excretion

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SUPPORTING INFORMATION AVAILABLE

Figures S1–S4. This material is available free of charge via the Internet at <http://pubs.acs.org>.

of 3-hydroxyisovaleric acid (3HIA).¹⁰ Increased 3HIA excretion reflects reduced activity of the biotin-dependent mitochondrial enzyme 3-methylcrotonyl-CoA carboxylase (MCC).¹⁰ Our interest in 3HIA-carnitine as a potential indicator of marginal biotin deficiency resulted from reports of newborns whose plasma 3HIA-carnitine concentrations were increased substantially above the upper limit of normal but did not ultimately prove to have a genetic abnormality such as multiple carboxylase deficiency¹¹ or genetic deficiency of MCC.^{12,13} In a previous report, we presented observations from a pilot study in which marginal biotin deficiency was induced experimentally in three healthy adults by feeding a low-biotin, high-egg-white diet for 4 weeks; mean plasma 3HIA-carnitine concentrations increased 3-fold.¹⁴ Conclusions from this pilot study were confirmed in a larger depletion and repletion study.¹⁵ These studies provide initial validation of plasma 3HIA-carnitine as an indicator of marginal biotin deficiency in humans and raise the possibility that urinary 3HIA-carnitine might also be an indicator of marginal biotin deficiency.

Here we describe the development and preliminary validation of a method for quantitation of the urinary concentration of 3HIA-carnitine. This urine method allows for high sample throughput and offers several advantages over the method for plasma 3HIA-carnitine. This urine method does not require expensive and time-consuming solid-phase or liquid-liquid extraction.

EXPERIMENTAL SECTION

Reagents and Chemicals

Optima LC/MS grade methanol was purchased from Fisher Scientific (Pittsburgh, PA). Reagent-grade trifluoroacetic acid was purchased in 1 mL ampules from Sigma Aldrich (St. Louis, MO). Deionized (DI) water used for this work was purified to 18.2 MΩ cm resistivity using a Siemans PURELAB Ultra laboratory water purification system (Warrendale, PA). Analytical standards of 3HIA-carnitine (>98% pure) and [*N-methyl*-D₃]-3-hydroxyisovaleryl carnitine (D3-3HIA-carnitine, >98% pure) were generous gifts from Cambridge Isotope Laboratories (Andover, MA).

Equipment

HPLC separations were performed using an Agilent Series 1200 quaternary liquid chromatography system (Santa Clara, CA). This HPLC system included an autosampler, high-pressure quaternary pumps, column oven, and system controller. Sample analysis was performed using an Applied Biosystems API-4000 QTRAP tandem mass spectrometer (Carlsbad, CA). A Precision Scientific heated water shaker/bath was used to warm urine samples to 60 °C. Urine samples were centrifuged using an IEC Centra CL3-R centrifuge (Needham Heights, MA).

Preparation of Analytical Standards, Quality Control Standards, and Subject Samples

All human urine, including subject samples and pooled human urine for quality control ("QC") standard preparations were thawed at room temperature, warmed to 60 °C in a water bath for 30 min, cooled to room temperature, and centrifuged at 3000g for 10 min to sediment urine precipitates as described previously.¹⁶ The supernatant was removed without disturbing the precipitate pellet. The human urine pool used to prepare QC standards was prepared by obtaining fresh untimed urine samples from five adult volunteers (four female, one male). A 40 mL aliquot from each of the five was pooled and the mixture vortexed for 30 s.

Analytical calibration standards and QC standards were prepared from a common 271 μmol/L aqueous stock solution of 3HIA-carnitine. A common aqueous stock solution of internal

standard (D3-3HIA-carnitine) was also prepared at 0.377 $\mu\text{mol/L}$. All analytical standard solutions were stored at -80°C until needed.

Daily working calibration standards were made fresh for each analytical run by first preparing a 3.81 $\mu\text{mol/L}$ intermediate working solution and then serially diluting in DI water to yield final concentrations ranging between 0.381 $\mu\text{mol/L}$ (100 ng/mL) and 0.00550 $\mu\text{mol/L}$ (1.56 ng/mL). QC standards were prepared by serial dilution in pooled human urine. Final exogenous 3HIA-carnitine concentrations were 10.7 (2800 ng/mL), 5.72 (1500 ng/mL), and 0.953 $\mu\text{mol/L}$ (250 ng/mL) and were representative of concentrations observed in subject urine samples. Before analysis, QC standards and subject samples were diluted 30-fold by addition of 10 μL of the respective sample to 290 μL of DI water and vortexed for 10 s. IS was added to all analytical standards, quality control standards, and subject samples by mixing 100 μL of standard or diluted QC standard or subject sample with 25 μL of the IS stock solution to yield a final IS concentration of 0.0754 $\mu\text{mol/L}$.

LC-MS/MS and Analytical Methods

The work described herein is a modification of a LC-MS/MS method we previously reported for plasma 3HIA-carnitine evaluations.¹⁴ The primary change included a selected reaction monitoring (SRM) information-dependent acquisition (IDA) experiment that acquired an enhanced product ion (EPI) spectra for 3HIA-carnitine present in each sample. Specific SRM and EPI parameters are summarized in Table 1. The IDA-SRM transition (262.2 $m/z \rightarrow 85.0 m/z$) threshold was set to an intensity of 500 counts per second. Representative IDA-EPI mass spectra are shown in the Supporting Information (Figures S1–S4).

Excretion rates for human urine samples were expressed as millimole (of 3HIA-carnitine) per mole of urinary creatinine. Creatinine was determined by the picric acid method as previously described.¹⁷

Clinical Study Design

The Institutional Review Board for the University of Arkansas for Medical Sciences approved this study. Written consent was obtained from each subject at enrollment, and consent was assessed intermittently throughout the study as part of the informed consent process.

To measure the effect of marginal biotin deficiency on the urinary excretion of 3HIA-carnitine, marginal asymptomatic biotin deficiency was induced in four healthy adults (one woman) by feeding a diet low in biotin and high in undenatured egg white for 28 days as previously described.¹⁸ A timed urine sample was collected for the 24 h prior to initiating the egg white diet (study day 0) and again on study day 28.

We have observed that marginally deficient individuals can appear spontaneously in normal populations, such as pregnant women.^{19–21} We and others have also documented that marginal biotin deficiency can develop in individuals chronically receiving anticonvulsant therapy.^{22–26} Accordingly, we speculate that exposure to other environmental factors might lead to marginal biotin deficiency in otherwise normal individuals. Consequently, in order to ensure biotin sufficiency on study day 0 of the biotin depletion phase, we instituted a loading and washout protocol before inducing deficiency as previously described.¹⁸ Briefly, biotin supplementation of 30 $\mu\text{g/d}$ (the “load”) was initiated on study day -21 ; on study day -14 , the biotin supplement was stopped (the “washout”).¹⁸ For the duration of the study, all subjects received a daily multivitamin that did not contain biotin.¹⁸ Biotin sufficiency on study day 0 and biotin deficiency on study day 28 were confirmed by PCC activity in PBLs

and urinary excretion of 3HIA. These were measured using methods described previously.^{9,10}

Statistical Methods and Reference Ranges

To assess the accuracy and precision of the method, the measured total 3HIA-carnitine concentration of the QC standards was corrected for the endogenous 3HIA-carnitine urine content. Therefore, the endogenous 3HIA-carnitine concentration in the urine was measured and subtracted from the measured total 3HIA-carnitine in the QC sample to give a calculated exogenous concentration of 3HIA-carnitine added. The accuracy was calculated as the percent relative error for the mean of the corrected QC concentrations using the following equation: $[(\text{corrected mean calculated concentration} - \text{nominal concentration}) / (\text{nominal concentration})] \times 100$. Analytical precision was calculated as the %CV for replicate measurements at the three QC concentrations. The limit of detection (LOD) was established at less than the lowest calibrator ($<0.00550 \mu\text{mol/L}$), and the lower limit of quantitation (LOQ) was calculated as three times the standard deviation of five replicate analyses of the low QC standard ($0.953 \mu\text{mol/L}$). The method reporting limit (MRL) applied a correction factor of 30 to the LLQ to account for the routine 30-fold dilution.

As previously reported,¹⁸ the normal range for PCC activity in PBLs was chosen as the 10th and 90th percentile of the distribution of PCC activity in 18 normal subjects rendered biotin sufficient by loading and washout. These percentiles, rather than ± 2.5 standard deviations, were chosen to define the normal range because the distribution of PCC activities (normalized by lymphocyte protein content) was not normal. The normal range was 320–750 pmol/(min · mg).

The normal range for the urinary excretion of 3HIA was chosen as the 10th and 90th percentiles from 54 normal subjects; this population included 18 healthy adults assured to be biotin sufficient by loading/washout and an additional 36 healthy adults who did not supplement their dietary biotin with other sources. The mean 3HIA excretion of the 36 individuals was not significantly different from the mean of 18 individuals. The normal range was 4.4–11.8 mmol/mol (urinary creatinine), as reported previously.⁹

For the loading and washout protocol used to ensure biotin sufficiency, data on plasma concentration of 3HIA-carnitine were available for only four subjects. Consequently, the reference range for plasma 3HIA-carnitine was chosen as the full range of those values.

Urine samples collected for this study were timed 24-h collections. However, urinary excretion rates for 3HIA-carnitine were expressed as millimole of 3HIA-carnitine per mole of urinary creatinine in order to preliminarily validate an index of biotin status that is practical for outpatient population studies. We have previously reported that the diagnostic utility of the two ways of expressing urinary 3HIA (by time and by creatinine) are similar.²⁷ No prior data were available for the urinary excretion of 3HIA-carnitine measured by this method. Consequently, the range for urinary excretion of 3HIA-carnitine was chosen as the full range of values at study day 0 (“biotin sufficiency”) of the subjects in this study.

For mean PCC activity, urinary 3HIA excretion, plasma 3HIA-carnitine concentration, and urinary 3HIA-carnitine excretion, the significance of differences from study day 0 to 28 was tested by Student's paired *t* test.

RESULTS AND DISCUSSION

Biotin is a water-soluble vitamin in the B complex and acts as an enzymatic cofactor for intracellular carboxylases.^{28–30} Biotin is released from digested dietary protein in the gut by

the enzyme biotinidase and incorporated into apocarboxylases by the enzyme holocarboxylase synthetase. There are five known mammalian carboxylases: pyruvate carboxylase (PC),³¹ propionyl-CoA carboxylase (PCC),⁹ 3-methylcrotonyl-CoA carboxylase (MCC),^{12,32} and the two isozymes of acetyl-CoA carboxylase (ACC).³³ These enzymes catalyze critical steps in several biochemical pathways, including gluconeogenesis, fatty acid biosynthesis, catabolism of branched chain amino acids, and odd chain fatty acid metabolism.²⁸

Marginal biotin deficiency reduces the enzymatic activity of MCC, thereby causing impairment in leucine catabolism. This metabolic disturbance causes increases in the intramitochondrial concentration of 3-methylcrotonyl-CoA. Accumulating 3-methylcrotonyl-CoA is converted to 3-hydroxyisovaleryl-CoA (3HIA-CoA) via an alternative pathway catalyzed by enoyl-CoA hydratase.¹² Acyl-CoAs such as 3HIA-CoA cannot cross the inner mitochondrial membrane. Two mechanisms are thought to allow the 3HIA moiety to leave the mitochondria: (1) deacylation by the enzyme acyl-CoA thioesterase to form the free organic acid 3HIA and diffusion across the inner mitochondrial membrane³⁴ and (2) formation of 3HIA-carnitine catalyzed by carnitine acetyltransferase and transport across the inner mitochondrial membrane by carnitine-acylcarnitine translocase.³⁵ These processes are thought to defend the intracellular free CoA/esterified CoA ratio and reduce the toxicity and other metabolic disturbances associated with elevated concentrations of acyl-CoAs.^{12,13,35,36}

The method described in this paper provides a reliable means for accurately and precisely measuring urinary concentration of 3HIA-carnitine, while providing sample throughput capacity designed to meet the demands of most clinical laboratories. The observed retention time for 3HIA-carnitine and D3-3HIA-carnitine was approximately 3.3 min in calibration standards and QC standards (Figure 1A) and in subject samples (Figure 1B). This retention time is consistent with that reported for the plasma method.¹⁴ Autosampler carryover was assessed using a blank DI water sample without the addition of any 3HIA-carnitine or IS. Analysis of this blank throughout method validation showed no autosampler carryover for either the unlabeled 3HIA-carnitine or IS.

Linear instrument response ($IR \equiv AUC_{Analyte}/AUC_{IS}$) over the calibration range was observed in all experiments, similar to our previous report.¹⁴ Independent calibration curves were produced for each batch by plotting the instrument response (IR) of each calibration standard against the nominal 3HIA-carnitine concentration. A least-squares linear regression without weighting was used to calculate a line of best fit. An example of a typical linear regression of IR versus $[3HIA-carnitine]/[D3-3HIA-carnitine]$ yielded the following equation: $IR = 0.0245\{[3HIA-carnitine]/[D3-3HIA-carnitine]\} + 0.000872$. The regression coefficient was 0.9992. The 95% confidence intervals (CIs) for the slope of the regression were 0.023–0.026, and the CIs for the y -intercept were –0.048 to +0.050. Slopes of the regression ranged from 0.022 to 0.029, y -intercepts ranged from +0.00087 to +0.0132, and regression coefficients ranged from 0.9992 to 0.9999. Precision and accuracy were assessed by replicate analysis ($n = 3–5$) of prepared QC standards. When endogenous 3HIA-carnitine ($0.522 \pm 0.0770 \mu\text{mol/L}$ for nine replicate measurements) was subtracted from QC standards, a high degree of accuracy and precision were observed (Table 2). No matrix effects were observed between prepared urine QC standards and aqueous calibration standards. LLQ and MRL measurements calculated as 0.114 and 0.347 $\mu\text{mol/L}$, respectively.

Our method for plasma 3HIA-carnitine requires solid-phase extraction prior to analysis.¹⁴ Potential removal of the SPE step would significantly improve sample throughput capacity and greatly reduce costs. To test the possibility, 20 diluted urine samples from subjects rendered marginally biotin deficient were analyzed both before and after SPE extraction.

The analytical results from samples not subjected to SPE extraction were plotted against the results for samples that were extracted using the reference SPE method (Figure 2). We observed a strong linear correlation ($r = 0.996$). Further, the regression was not significantly different from the line of identity as judged by the CIs for the slope incorporating 1 (0.946–1.033) and the CIs for the y -intercept incorporating the origin (–0.064 to +0.154). Comparison of the two methods (dilution only vs SPE reference method) for analysis of urine samples justifies omission of solid-phase extraction.

To examine the validity of urinary 3HIA-carnitine as an indicator of marginal biotin deficiency, four subjects were experimentally rendered marginally biotin deficient. All subjects were biotin sufficient on study day 0 as judged by at least two of three indicators of biotin status: PCC activity (Figure 3A), urinary excretion of 3HIA (Figure 3B), and plasma 3HIA-carnitine (Figure 3C). Failure of all subjects to fall within the normal ranges at study day 0 likely reflects either the statistical process of defining the normal range, limited sample size, or the diagnostic sensitivity of any given single indicator of biotin status. By study day 28, marginal biotin deficiency was successfully induced in all four subjects as judged by abnormally decreased PCC activity (Figure 3A), abnormally increased urinary excretion of 3HIA (Figure 3B), and abnormally increased plasma concentration of 3HIA-carnitine (Figure 3C).

Mean urinary excretion of 3HIA-carnitine for these four subjects increased 3.5-fold ($p = 0.026$) between study day 0 and 28 (Figure 4). The observed increases in the excretion of 3HIA-carnitine paralleled the increases in the urinary excretion of 3HIA and plasma 3HIA-carnitine concentration.

CONCLUSION

Validated indicators of biotin status include PCC activity from PBLs isolated from venous blood and 3HIA excretion in urine.^{9,10} These traditional assays present significant analytical challenges. The fundamental principle of the lymphocyte PCC assay is quantitation of the catalysis by PCC of ¹⁴C incorporation from ¹⁴C-bicarbonate into ¹⁴C-labeled methylmalonyl-CoA. The PCC must be enzymatically active after sample processing, cell isolation, and storage. Accordingly, the blood samples require special handling, the lymphocytes must be carefully isolated from peripheral blood, and the cell isolates must be stored frozen. Hence, the unavoidable unpredictability of timing of sample availability for certain populations (e.g., cord blood) is potentially problematic for the PCC assay. Moreover, care must be taken during the processing and isolation steps of sample preparation because any hemoglobin contamination from red blood cells in the lymphocyte pellet interferes with the normalization of the PCC activity by lymphocyte protein. Likewise, clean separation of the ¹⁴C in the reaction product (¹⁴C-labeled methylmalonyl-CoA) from the ¹⁴C-bicarbonate substrate and maintenance of a stable specific activity of ¹⁴C-bicarbonate due to bicarbonate volatility are technically demanding. In general, when stored at –20 or –80 °C, acylcarnitines are more stable than PCC activity. Measurement of 3HIA excretion in urine is complicated and involves liquid-liquid extraction and derivatization prior to GC–MS analysis.¹⁰ Further, silylation reagents and their derivatives decompose when exposed to moisture.

The method presented here extends previous work in plasma providing evidence that urinary 3HIA-carnitine can be accurately and precisely measured using LC–MS/MS without extensive sample preparation. This report also provides the first preliminary evidence that urinary 3HIA-carnitine is an indicator of marginal biotin deficiency. Further validation in larger populations is needed to confirm this initial conclusion. Nonetheless, the analytical method presented in this report may potentially provide flexibility in the determination of

biotin status in humans subjected to a variety of phenotypical factors (e.g., pregnancy, diet, anticonvulsant therapy, medications, disease, and smoking) under a variety of clinical circumstances that have the potential to alter biotin status.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the following agencies: National Institutes of Health, grants R37 DK36823 (D.M.M.), R37 DK36823-26S1 (D.M.M.), and R01 DK79890-01S1 (DMM); Arkansas Biosciences Institute, Arkansas Tobacco Settlement Proceeds Act of 2000 (D.M.M.); Centers for Disease Control contract, 200-2007-21729 (J.H.M.); Bioterrorism Cooperative Agreement, U90/CCU616974-07 (J.H.M.). The project described was supported by Award Number 1UL1RR029884 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Research Resources or the National Institutes of Health. We thank Ms. Brandy VanDenBerg at UAMS for editorial support for this work. We thank Joel Bradley, Ron Trolard, and Rosemarie Bachand of Cambridge Isotope Laboratories for the generous gift of the authentic 3HIA-carnitine and D3-3HIA-carnitine used in the work described herein.

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Figure 1.

(A) Chromatogram for a 10.7 $\mu\text{mol/L}$ (2800 ng/mL) 3HIA-carnitine QC standard prepared in human urine (3HIA-carnitine, solid line; D3-3HIA-carnitine, dotted line). (B) Chromatogram for a representative subject sample on study day 0 (3HIA-carnitine, solid line; D3-3HIA-carnitine, dotted line).

Figure 2. Correlation of urinary 3HIA-carnitine results obtained using the dilution method and the reference SPE method.

Figure 3. Biochemical response to a biotin-depleting diet for four subjects at study day 0 (biotin sufficient) and study day 28 (rendered biotin deficient): (A) PCC activity in PBL, (B) urinary excretion of 3HIA, and (C) plasma concentration of 3HIA-carnitine.

Figure 4. Urinary 3HIA-carnitine excretion for the four human research subjects at study day 0 and 28.

MS/MS Experimental Conditions for Specific Reaction Monitoring (SRM) and Information-Dependent Acquisition-Enhanced Product Ion (IDA-EPI)

Table 1

MS/MS experiment	analyte	Q1 (m/z)	Q3 (m/z)	CE ^a (V)	EP ^b (V)	DP ^c (V)	CXP ^d (V)
SRM	3HIA-carnitine	262.2	85	35	10	71	8
	D3-3HIA-carnitine	265.2	85	33	10	71	10
IDA-EPI	3HIA-carnitine	262.2	50-300	30	10	71	11

^a Collision energy.

^b Entrance potential.

^c Declustering potential.

^d Collision cell exit potential.

Table 2Mean Concentration^a, Precision^b, and Accuracy^c for Prepared Diluted^d QC Standards

<u>concentration (μmol/L)</u>			
nominal	mean calcd	precision (%CV)	accuracy (%RE)
0.953 (<i>n</i> = 5)	0.998	11.6	4.70
5.72 (<i>n</i> = 5)	5.94	6.22	3.79
10.7 (<i>n</i> = 3)	11.3	3.60	5.80

^aCalculated from the linear least-squares regression composed for the calibration standards (*n* = 6).^bCalculated as the coefficient of variation for the QC standard (*n* = 3–5) concentrations after correction for endogenous content of 3HIA-carnitine in the plasma.^cCalculated as [(corrected mean calculated concentration – nominal concentration)/nominal concentration] × 100.^dDilution factor = 30.